A HANDBOOK OF PLANT NUTRITION- 2ND EDITION

COPPER

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1 HISTORICAL BACKGROUND

Copper (Cu) is a $3d^{10}$ transition metal that belongs to Group IB. It has an atomic number of 29 and an atomic mass of 63.546 amu. The melting point: is 1083.0 °C (1356.15 K, 1981.4 °F), boiling point: 2567.0 °C (2840.15 K, 4652.6 °F) and density at 293 K is 8.96 g cm^{-3}. Cu is a redox-active transition metal that can exist as Cu^{2+} (Cu(II), cuprous) and Cu^{+} (Cu(I), cupric) ions. A single electron in the outer 4s orbital ($4s^1$) confers this electronic characteristic, and this $4s^1$ electron is difficult to remove. The first and second ionization potentials are 7.72 eV and 20.29 eV, respectively. The fact that the second ionization potential is much higher that the first one means that several Cu^{+} species can exist. Cu is one of the few metals that can be found in nature in its elemental form, although the quantities in which is found in its free state are very small.

Cu is an essential element for all living organisms since it is a component of a variety of Cu-containing proteins involved in biological reactions such us oxidation of iron (Fe), insertion of oxygen in organic substrates, disproportionation of oxygen free radicals. These reactions are important in

redox electron transport chains and oxidative stress responses. In particular, in plants Cu functions in photosynthesis and respiratory electron transport and also it is important for cell wall metabolism, detoxification of oxygen species, ethylene sensing, and synthesis of polyphenols. The biological role of Cu started to be relevant in biology with the accumulation of oxygen in the atmosphere and the oceans, which changed the earth from aerobic to anaerobic conditions (Crowe et al., 2013). The development of primitive oxygenic photosynthetic organisms (*i.e.* cyanobacteria), ancestors of bryophytes and land plants, was responsible for this environmental change. This fact led to a decrease in Fe solubility and a shift from the use of Fe towards Cu in similar biological systems (Crichton and Pierre, 2001; Yruela, 2013).

Cu has diverse roles in biology due to its redox properties, which led it to participate in numerous interactions with proteins to drive diverse structures and reactions. Cu^{+} has affinity for thiol and thioether groups (found in cysteine or methionine amino acids), and Cu^{2+} normally exhibits coordination to oxygen or imidazole nitrogen groups (found in aspartic and glutamic acid, or histidine, respectively). According to spectroscopic and magnetic features the Cu centers in proteins have been classified as type I, type II, type III, binuclear Cu_A and binuclear Cu_B centers. In type I centers (also named blue copper centers) the Cu ion is coordinated to nitrogens (N) of two histidine (His) residues and sulfurs (S) of a cysteine (Cys) and a methionine (Met) (or oxygen (O) of a glutamine (Gln)), exhibiting a square planar coordination. It shows an intense absorption maximum at around 600 nm and narrow hyperfine splittings in electron paramagnetic resonance (EPR) spectroscopy. In plants, plastocyanin and multicopper oxidases belong to this type of Cu-containing protein. Type II

centers (also named non-blue copper centers) are formed by only one Cu atom. They do not give strong absorption at 600-700 nm since they lack S atoms as ligands, and show larger hyperfine coupling constants in the EPR spectrum. In this case the Cu ion is coordinated to four or five ligands, which can be N atoms of His residues, and/or O atoms of tyrosine (Tyr) residues and water molecules. They exhibit a square planar coordination. Normally, these centers are present in enzymes involved in oxidations or oxygenations. In plants, copper-zinc superoxide dismutase (Cu/Zn-SOD) and amine oxidase belong to this group. Type III Cu-centers are constituted by two Cu atoms each coordinated to N atoms of three His residues. It is not detected in the EPR spectrum because of the exchange interaction between the two Cu^{2+} (S = ½) metal ions caused by bridging ligation, leading to strong antiferromagnetic coupling. They play important roles in O_2 binding, activation, and reduction to H_2O. In plants, enzymes such as catechol oxidase, ascorbate oxidase, and laccase contain this type of Cu-center.

The Cu_A and the Cu_B centers are characterized by a binuclear and mononuclear geometry, respectively. In the binuclear Cu_A center two Cu atoms are bound by two S atoms of Cys residues and each is bound to one N atom of a His residue. The coordination is completed with an O atom from a glutamic acid (Glu) residue and an S from a Met residue. In the Cu_B center the Cu atom is coordinated to three His ligands in trigonal pyramidal geometry. In plants cytochrome c oxidase is an enzyme that contains both Cu_A and Cu_B centers. Finally, the tetranuclear copper Z center (CuZ), found in nitrous-oxide reductases, is constituted by four Cu atoms coordinated by seven His residues and bridged by an S atom.

In the redox process between Cu^{+} and Cu^{2+} states, Cu ions can generate harmful hydroxyl radicals and other reactive oxygen species *via* Haber-Weiss and Fenton reactions (Halliwell and Gutteridge, 1984; Yruela et al., 1996). These can damage proteins, nucleic acids, and lipids and interfere with the synthesis of [Fe-S] clusters or the activity of enzymes. On the other hand, ROS have a physiological role in signalling and regulatory processes but the details of the relationship between Cu sensing mechanisms and ROS sensing mechanisms in plants are so far poorly understood (Ravet and Pilon, 2013).

2 UPTAKE OF COPPER BY PLANTS

Cu availability is a prerequisite for plant growth and development. The Cu content of soils ranges from 2 to 100 mg kg^{-1}, with an average value of *ca.* 30 mg kg^{-1}, but most of this is not available for plants. Cu concentration in plant tissues varies depending on plant species or ecotypes, developmental stage and environmental factors such as nitrogen supply and soil chemical properties (Ginocchio et al., 2007). For instance, Cu availability in soils decreases above pH 7.0 due to slightly basic pH conditions favoring the binding of Cu to soil chemical components. On the contrary, Cu availability increases under acidic soil conditions due to the increase of Cu ions in the soil solution (Kopsell and Kopsell, 2006). Nitrogen supply can also affect the availability of Cu (Jarvis and Whitehead, 1981). Conversely, the presence of Cu can affect the development of some legume symbioses, and the effectiveness of nitrogen fixation and the productivity of legumes (O'Hara, 2001).

Normally, the Cu concentration in plant tissues is between 2 and 50 μg g^{-1} dry weight (Epstein and Bloom, 2005; Marschner, 2012) and the average

composition of Cu in leaves is 10 μg g^{-1} dry weight (5-20 μg g^{-1} dry weight) (Baker and Senef, 1995) but these concentrations can vary among plant species and varieties. The amount of free Cu ions in the cytosol is probably limited to less than one ion per cell (Rae et al., 1999). Cu-deficient plants (< 5 - 20 μg g-1 in vegetative tissue) exhibit reduced growth and development, with reproductive organs and youngest leaves displaying the most severe symptoms (Burkhead et al., 2009). The rate of copper uptake in plants is low in comparison with other essential micronutrients (Kabata-Pendias and Pendias, 1992). This is partly explained by the fact that *i)* plants grown under high nitrogen supply require significantly more Cu; *ii)* the bioavailability of Cu tends to be greater in acidic soils, meaning that in most agricultural soils it is comparatively low.

Cu concentrations in cells need to be maintained at low levels since the element is extremely toxic because of its high redox properties. The critical free Cu concentration in nutrient media (below which Cu deficiency occurs) ranges from 10^{-14} to 10^{-16} M. Plants usually find a variable supply of Cu in the soil since typically soil solution concentrations range from 10^{-6} to 10^{-9} M (Marschner, 2012), but plants may still need to solubilize and reduce the metal.

Concentrations of free metal ions or metal chelates in the soil solution are generally rather low, although they depend on soil properties (Kochian, 1991; Marschner, 2012). In both soil solution and the solid phase Cu is mainly associated with inorganic and organic matter by complexation or adsorption. Cu ions have a high affinity for binding sites of soil components, and they can also be adsorbed onto surfaces of clays and Fe or Mn oxides, co-precipitated with carbonates and phosphates or be present in the lattice of primary silicate

minerals. Cu ions can be also bound to cell walls and to the outer membrane surface of plant root cells. The distribution of Cu among these various solid and plant components will greatly influence the chemical mobility and hence the amount of Cu potentially taken up by plants. At acidic pH, dissolved Cu increases because of its weaker adsorption and the free Cu ion activity is higher. Additionally, with increasing pH, competitive adsorption arises between organic matter in the solid phase and dissolved organic carbon, generally leading to an increase in Cu concentration in the soil solution due to a higher dissolved organic carbon content (Carrillo-González and González-Chávez, 2006). Thus, upon increasing pH, the Cu ions activity considerably decreases at the expense of organically bound complexes species in the soil solution (Sauvé et al., 1997).

On the other hand, in the rhizosphere, root and microbial activities can influence the chemical mobility of metal ions and ultimately their uptake by plants as a consequence of alterations of soil pH or dissolved organic carbon content (Hinsinger and Courchesne, 2007). For instance, in the case of Graminaceous species, the increased root secretion of Fe-chelating compounds (phytosiderophores) under Fe deficiency has been reported to increase Cu uptake in a calcareous soil (Chaignon et al., 2002b). It is noticeable that soil chemical properties can differ between the bulk soil and the rhizosphere, so considering only properties in the bulk soil might be a poor predictor of Cu bioavailability and ultimately Cu uptake, which is influenced by the particular properties induced in the rhizosphere by roots. Accordingly, contradictory results concerning the effect of pH on Cu uptake by plants are found in the literature. In very acidic soils, plant Cu concentration increased compared with

calcareous soils in rape (*Brassica napus* L.) and tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) (Chaignon et al., 2002a; Chaignon et al., 2003; Cornu et al., 2007). In contrast, Cu accumulation in maize (*Zea mays* L.) was as high in calcareous soils as in acidic soils (Brun et al., 2001). Michaud et al. (2007) did not find a clear relationship between Cu uptake and soil pH in durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.) in Cu-contaminated soils, probably due to the implication of root-induced changes of pH and dissolved organic carbon in the rhizosphere. At low pH, alkalization was observed in the rhizosphere compared with the bulk soil, which may result in a reduced Cu bioavailability. In calcareous soils, a larger chemical mobility may be related to phytosiderophore secretion leading to greater Cu uptake by plants.

The molecular mechanisms of Cu mobilization and uptake by roots from soil solutions remain unclear. Fe reduction and complexation mechanisms have been shown to affect Cu speciation in controlled environments. For instance, ferric reductase oxidase 2 (FRO2) is capable of reducing Cu^{2+} and FRO3 is up-regulated during Cu deficiency (Robinson et al., 1999; Burkhead et al., 2009; Palmer and Guerinot, 2009; Bernal et al., 2012). The fractionation of stable copper isotopes ($^{65}Cu/^{63}Cu$) during uptake into plant roots and translocation to shoots can provide information on Cu acquisition mechanisms (Ryan et al., 2013). The heavier isotope was preferentially translocated to shoots in *Solanum lycopersicum,* tomato, (strategy I plant) whereas *Avena sativa* L.*,* oat, (strategy II plant) showed no significant fractionation during translocation, with no effect of Fe supply in either species. The majority of Cu in the roots and leaves of both species existed as sulfur-coordinated Cu^{+} species resembling glutathione/cysteine-rich proteins. The presence of isotopically light Cu in *S.*

lycopersicum is attributed to a reductive uptake mechanism, and the isotopic shifts within various tissues are attributed to redox cycling during translocation. The lack of isotopic discrimination in *A. sativa* plants suggested that Cu uptake and translocation are not redox selective.

The Cu concentration in shoots of plants varies considerably between species (Beeson et al., 1947; Thomas et al., 1952; Alloway, 2008).This suggests different abilities to either absorb Cu from the soil, or translocate Cu from root to shoot. The factors that allow some plants to take up more Cu than others are unclear. Low uptake of Cu into shoots of plants is partly due to Cu being strongly bound to soil organic matter or partly because Cu remains in the roots in high amounts. In both cases Cu is poorly translocated to the shoot (Jarvis and Whitehead, 1981; Whitehead, 1987). On the other hand, divalent cations can alter the permeability of the plasma membrane depending of their concentration, and affect the trans-root potential and H^+ efflux of excised roots (Kennedy and Gonsalves, 1987). One of the physiological responses to excess Cu is K^+ efflux from the roots, which is interpreted as a symptom of toxicity resulting from Cu-induced oxidative damage to the plasma membrane. Cu retention by roots impairs Cu translocation to xylem and shoots and increases the risks of membrane damage in the roots themselves.

3 PHYSIOLOGICAL RESPONSES OF PLANTS TO SUPPLY OF COPPER

Cu is a micronutrient, but is highly phytotoxic above micromolar concentrations (Marschner, 2012; Yruela, 2005; Yruela, 2008) and consequently induces physiological responses when supplied in excess. Cu-derived chemicals have been used as broad-specificity fungicides since 1882, when the French botanist

and mycologist Pierre-Marie-Alexis Millardet developed a formulation, consisting of hydrated lime (calcium hydroxide), copper sulfate and water (Bordeaux mixture), which controlled and protected the vineyards of France infested by the destructive *Phylloxera*. It was subsequently used on late potato blight caused by *Phytophthora infestans* (the agent of the Irish potato famine) (Large, 1940; Dixon, 2004). It was the first fungicide to be used worldwide and this agronomic practice can increase the Cu concentration in soils (Lepp et al., 1984; de Loland and Singh, 2004; Pietrzak, 2004; Fan et al., 2011; Ruyters et al., 2013; Wightwick et al., 2013).

Cu phytotoxicity symptoms are normally characterized by interveinal chlorosis, reduction of root and shoot volume, reduction of stem size and leaf size (Ouzounidou et al., 1995; Prasad and Strzalka, 1999; Kopsell and Kopsell, 2006; Marschner, 2012; Yruela, 2005; Yruela, 2008). It is noted that this toxicity is dependent on plant species, concentration of metal supplied, exposure time, culture conditions and soil properties (Rooney et al., 2006; Li et al., 2010). Differences in Cu phytotoxicity on vineyard and cereal (barley, maize, rice) crops grown in acidic and calcareous soils have been reported (Delas, 1963; Reichman, 2002; Michaud et al., 2007; Guo et al., 2010; Li et al., 2010). Normally, Cu toxicity decreases as soil pH increases. Guo *et al*. (2010) reported that the critical concentration of Cu added to soils that decrease maize grain yield by 10% are higher (711 mg kg^{-1}) for calcareous soil with a pH of 8.9 than for acidic soil (23 mg kg^{-1}) with a pH of 5.3. Rhizosphere alkalisation can restrict Cu bioavailability in acidic soils. Bravin *et al.* (2009) found that Cu bioavailability was 2.4- to 4.2-fold higher when durum wheat plants were fed with mixed NH_4^+ - NO_3^- compared with plants fed NO_3^- alone.

The early effect of Cu toxicity in plants is rhizotoxicity (Marshner, 2012; Sheldon and Menzies, 2005; Kopittke and Menzies, 2006). Copper translocation towards shoots is efficiently restricted by the large accumulation of Cu in roots. Thus, important Cu rhizotoxicity and deleterious physiological effects (*i.e.* altered root growth and nutrient uptake) are expected to occur before shoot Cu concentration reaches abnormal values. To evaluate ecotoxicological risks caused by excess Cu, simple and sensitive indicators of Cu phytotoxicity based on early and primarily Cu rhizotoxic effects are necessary. Few references of such Cu phytotoxicity are available in the literature and most of these were established for shoots (*i.e.* critical Cu concentration), not for roots, probably due to difficulties encountered in measuring Cu concentration in roots of soil-grown plants because of potential contamination of roots with soil particles (Beckett and Davis, 1978; MacNicol and Beckett, 1987; Reuter and Robinson, 1997; Kopittke and Menzies, 2006).

Some of these responses are common to Cu deficiency, and this makes it difficult to distinguish the origin of such symptoms. Both Cu deficiency and Cu toxicity cause changes in the expression of genes, which activate morphological changes in the plant, mainly concerning root and leaf architecture. Altogether this reduces plant biomass and crop productivity.

4 GENETICS OF ACQUISITION AND DISTRIBUTION OF COPPER BY PLANTS

Plants are characterized by a vascular transport system that requires a complex Cu homeostasis machinery, and which provides the metal to Cu-dependent proteins in both chloroplasts and mitochondria. Different types of transporter

proteins involved in Cu uptake and distribution have been identified, based on their homology with bacteria, yeast and mammals. These transporters include both integral membrane proteins and soluble proteins named metallochaperones. Examples are: *i)* the high-affinity Cu transport proteins named COPTs in plants (Ctr in yeast, *Saccharomyces cerevisiae*, and humans) (Kampfenkel et al., 1995; Puig and Thiele, 2002; Pole and Schützdendübel, 2004); *ii)* the P-type ATPase pump transporters (William et al., 2000; Williams and Mills 2005; Yruela 2005; Yruela 2008); *iii*) the ZIP zinc (Zn) transporter family (Puig et al. 2007); *iv)* Yellow-Stripe-Like (YSL) protein family; *v)* copper chaperones (Table 1).

4.1 COPT transporters

Cu enters the cytosol of plant cells mediated by members of the Copper Transporter Protein (COPT) family of transporters. The COPT proteins share 35% to 64% sequence identity and 47% to 73% sequence similarity with each other and have a similar structure to COPT/Ctr proteins in other species. Members of this family have been investigated at the molecular and functional level in *Arabidopsis* (Puig and Thiele, 2002; Puig et al., 2007) and *Oryza sativa* L., rice (Yuan et al., 2011). In the *Arabidopsis* genome, six members of the COPT/Ctr family (*COPT1-6*) have been identified (Sancenón et al., 2003; Peñarrubia et al., 2010; Perea-García et al., 2013). In *O. sativa* the COPT/Ctr family consists of seven members (*COPT1-7*) (Yuan et al., 2011). They could act alone or cooperatively to mediate Cu transport in different plant tissues.

All transporters belonging to the COPT/Ctr family contain three predicted transmembrane (TM) segments and most possess an N-terminus methionine-

and histidine-rich putative metal binding domain localized in the extracellular space. The C-terminus and the loop between TM1 and TM2 segments are exposed to the cytosol (Puig and Thiele, 2002; Klomp et al., 2003). Genetic data and *in vivo* uptake experiments have demonstrated that an extracellular methionine residue, located approximately 20 amino acids before TM1, and a MxxxM motif within TM2, are essential for Cu acquisition, and probably mediate metal coordination during transport. The structure of human Ctr1 transporter, a homolog of COPT proteins, in a phospholipid bilayer has shown a compact and symmetrical trimer organization with a novel channel-like architecture where a conserved GxxxG motif within TM3 is essential for trimerization (Aller et al., 2004; Aller and Unger, 2006). The expression of members of the COPT/Ctr gene family is controlled by environmental Cu level in different species. In general, they are transcriptionally up-regulated in response to Cu deficiency and down-regulated in response to Cu excess.

COPT1 and COPT2 localized in the plasma membrane are involved in Cu acquisition and transport toward the cytosol (Sancenón et al., 2003; Andrés-Colás et al., 2010), and are highly specific for Cu^{+} ions. COPT1 is activated under Cu-limiting conditions, and regulates root elongation and pollen development. COPT1 plays a predominant role in Cu uptake from soil through the root tips (Sancenón et al., 2004). *COPT1* expression in roots has been corroborated in transgenic plants where the COPT1 promoter drives GUS expression, and it has been assigned to specific peripheral cells in a limited narrow root apical zone. Reduction of Cu uptake was observed in *COPT1* antisense plants. COPT2 participates in the attenuation of Cu deficiency responses driven by Fe limitation, possibly to minimize further Fe consumption.

In *Arabidopsis*, *COPT2* expression is up-regulated in roots by both Cu and Fe deficiencies (Sancenón et al., 2003; Colangelo and Guerinot, 2006; Waters et al., 2012; Perea-García et al., 2013) indicating links between Cu and Fe transport in *Arabidopsis thaliana*. Furthermore, *AtCOPT1* and *AtCOPT2* genes display a similar expression pattern under slight Cu deficiency in several plant aerial tissues and organs (including cotyledons from young seedlings, trichomes, anthers, and mature pollen), suggesting a partial functional redundancy (Perea-García et al., 2013). On the contrary, *AtCOPT1* and *AtCOPT2* present notable differences in root expression patterns. *AtCOPT1* is exclusively expressed in primary and secondary root tips whereas *AtCOPT2* is expressed in sub-apical root regions, indicating local and specific functions and signalling in roots. Moreover, both *AtCOPT1* and *AtCOPT2* differ in their response under Fe deficiency. In these conditions, *AtCOPT1* is down-regulated in restricted Cu medium and *AtCOPT2* is up-regulated. Additionally, a role of AtCOPT2 transport activation has been reported in Pi starvation conditions. Connections between Pi starvation responses and the regulation of other metal ion transporters have been suggested (Abel, 2011; Chiou and Lin, 2011).

The AtCOPT3 and AtCOPT5 proteins are involved in intracellular Cu distribution. In particular, AtCOPT5 participates in the mobilization of Cu from the vacuole or prevacuolar compartments towards the cytosol under extreme Cu-deficiency (García-Molina et al., 2011; Klaumann et al., 2011). AtCOPT6 is localized in the plasma membrane, interacts with AtCOPT1 and regulates the response to either Cu limitation or excess (Jung et al., 2012). Its transcript is highly expressed in leaves and is up-regulated under Cu limitation indicating that probably its role consists in maintaining optimum Cu level for the

photosynthetic apparatus. In *O. sativa* it has been described that COPT2, COPT3 and COPT4 physically interact with COPT6 indicating that they may cooperate with COPT6 for Cu transport. COPT7 would function alone in different rice tissues, except in root (Yuan et al., 2011). The *OsCOPT2*, *OsCOPT3, OsCOPT4* and *OsCOPT6* genes are all expressed in stem, sheath, leaf and panicle tissues.

4.2 P_{1B}-type ATPase pump transporters

The P-type heavy metal ATPases (HMAs) are a subgroup of the large superfamily of P-type ATPases, which use ATP to pump a variety of charged substrates across biological membranes and are distinguished by the formation of a phosphorylated intermediate during the reaction cycle. This family of transporters (HMAs) is diverse in terms of tissue distribution, subcellular localization, and metal specificity. In plants, at least eight members have been identified in *A. thaliana.* The *O. sativa* genome contains nine P1B-type ATPase genes and ten members of this subfamily have been identified in barley (*Hordeum vulgare* L.). The P-type heavy metal ATPases are implicated in the transport of a range essential and potentially toxic metals (*i.e.*, Cu^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}) across cell membranes. Solioz and Vulpe (Solioz and Vulpe, 1996) defined the P-type heavy metal ATPases as CPx-ATPases because they share the common feature of a conserved intramembranous cysteine-proline-cysteine, cysteine-proline-histidine or cysteine-proline-serine motif (CPx motif) which is thought to function in heavy metal transduction. Structurally, P_{1B}-type ATPases contain eight transmembrane (TM) segments with various cytoplasmic domains involved in enzyme phosphorylation (P-domain), nucleotide binding (N-domain)

and energy transduction (A-domain), domains that are common for all P-type ATPases. Additionally, P_{1B}-ATPases show different features associated with their singular function in heavy metal transport (Argüello, 2003; Argüello et al., 2007).

Functional studies of HMAs have shown that these transporters can be divided into two subgroups based on their metal-substrate specificity: a copper (Cu)/silver (Ag) group, which transport monovalent cations and a zinc (Zn)/cobalt (Co)/cadmium (Cd)/lead (Pb) group, which is involved with divalent cations. In *Arabidopsis* AtHMA1 to AtHMA4 are divalent cation transporters, involved in the export of Cu^{2+}, Zn^{2+} and Cd^{2+}. In contrast AtHMA5 to AtHMA8 act in transport of monovalent Cu^{+} ions. In *O. sativa* OsHMA1 to OsHMA3 belong to the divalent Zn/Co/Cd/Pb subgroup (Takahashi et al., 2012a). The first member cloned in plants was PAA1(AtHMA6) (P1B-type ATPase of *Arabidopsis* 1) (Tabata et al., 1997), which is responsible for the delivery of Cu to chloroplasts and provides the cofactor for the stromal Cu/Zn-SOD enzyme and for the thylakoid lumen protein plastocyanin, two proteins involved in antioxidant enzymatic activity and photosynthetic electron transport function, respectively (Shikanai et al., 2003). PAA2(AtHMA8), closely related to PAA1(AtHMA6), transports Cu into the thylakoid lumen to supply plastocyanin (Abdel-Ghany et al., 2005). PAA1(AtHMA6) is expressed in both roots and shoots, while PAA2(AtHMA8) is only detected in shoots. A double *paa1paa2* mutant resulted in seedling lethality, a more severe phenotype than that observed for plants defective for either gene separately, underlying the importance of Cu to photosynthesis (Weigel et al., 2003; Abdel-Ghany et al., 2005). The homolog of PAA2(AtHMA8) in soybean (*Glycine max*), named

GmHMA8, was identified and localized in the thylakoid membrane (Bernal et al., 2007b). AtHMA1 is localized in the chloroplast envelope and contributes to the detoxification of excess Zn and Cu (Seigneurin-Berny et al., 2006; Kim et al., 2009). AtHMA3 is localized in the vacuolar membrane and plays a role in detoxifying Zn and Cd through vacuolar sequestration (Gravot et al., 2004; Morel et al., 2009). HMA3 is recognized as the major locus responsible for the variation in leaf Cd accumulation in *A. thaliana* (Chao et al., 2012). AtHMA2 and AtHMA4 are localized in the plasma membrane and function in Zn and Cd efflux from cells (Mills et al., 2003; Eren et al., 2004; Hussain et al., 2004; Mills et al., 2005; Verret et al., 2005). AtHMA2 and AtHMA4 are expressed in tissues surrounding the vascular vessels of roots (Hussain et al., 2004; Verret et al., 2004). Additionally, it has been suggested that HMA4 acts in Zn loading to the xylem and that HMA2 and HMA4 could be involved in Cd translocation in *A. thaliana* (Wong and Cobbett, 2009; Mills et al., 2010). Furthermore, HMAs are involved in metal hyperaccumulation and hypertolerance. In *Arabidopsis halleri*, greater HMA4 expression in the roots contributes to the high efficiency of Zn translocation from roots to shoots (Talke et al., 2006; Courbot et al., 2007). Furthermore, expression of *HMA4* contributes to Zn and Cd hyperaccumulation in *A. halleri* and *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) (Ó Lochlainn et al., 2012). NcHMA4/TcHMA4 is present in vascular tissue and is thought to function in metal distribution (Craciun et al., 2012). TcHMA3 in the leaves also contributes to Cd hyperaccumulation and hypertolerance via the high sequestration of Cd in leaf vacuoles (Ueno et al., 2011).

The Responsive to Antagonist (RAN1)/AtHMA7 transporter is responsible for the biogenesis of ethylene receptors by delivering Cu to ETR1 through the

endoplasmic reticulum, where it is required for the formation of functional ethylene receptors (Woeste and Kieber, 2000; Chen et al., 2002). The plant hormone ethylene is an important signal in many abiotic stress situations and also in plant pathogen interaction. RAN1(AtHMA7) has also been found in *Brassica napus* (BnRAN1) (Southron et al., 2004). Among the rice P_{1B}-ATPases, OsHMA9 was found to form a subclass with RAN1(AtHMA7), which might transport Zn, Cd, and Pb, although OsHMA9 belongs to the Cu/Ag subgroup phylogenetically (Lee et al., 2007). It plays a role in Cu detoxification, acting as an efflux pump in the plasma membrane (Sichul et al., 2007). Mutant *oshma9-1* and *oshma9-2* plants exhibited the phenotype of increased sensitivity to high levels of Cu, and also Zn and Pb. The *OsHMA9* gene was mainly expressed in vascular tissues, including xylem and phloem and weakly expressed in mesophyll tissues. In developing tissues, expression was strong in anthers, suggesting a putative role in metal delivery to rice anthers. The importance of metal transport in anthers has been previously reported. The AtHMA5 transporter, the closest homolog of RNA1(AtHMA7) in the P_{1B}-type ATPase subfamily, is strongly and specifically induced by Cu in whole plants. The *hma5* T-DNA insertion mutants are hypersensitive to Cu and HMA5-defective plants accumulate Cu in roots to a greater extent than wild-type plants, suggesting its key role in transmembrane transport, and particularly in root Cu detoxification (Andrés-Colás et al., 2006). This phenotype is the opposite of that observed for the COPT antisense lines, supporting the notion that COPT1 and AtHMA5 transport Cu in opposite directions. AtHMA5 is mostly expressed in roots, flowers and pollen. The specific interaction of AtHMA5 with two different ATX1-type chaperones, ATX1 and CCH, in *Arabidopsis* has been

demonstrated. Although further experiments are necessary to confirm the fact, it has been proposed that AtHMA5 could be involved in Cu efflux from specific root cells and its overexpression in plants could be a strategy for improving Cu detoxification under Cu excess.

OsHMA3 transports only Cd and plays a role in the sequestration of Cd into vacuoles in root cells (Ueno et al., 2010; Miyadate et al., 2011). On the contrary, there is little information on the role of OsHMA1 which is thought to be involved in Zn transport. *OsHMA1* expression is highly up-regulated by Zn deficiency in shoot tissue (Suzuki et al., 2012). *OsHMA1* is highly expressed in the leaf blade, but is also expressed in the root, inflorescence, anther, pistil, lemma, palea, ovary, embryo, and endosperm. This suggests that OsHMA1 plays a role in Zn transport in the entire plant through all growth and developmental stages. OsHMA1, just like AtHMA1, may play a role in Zn efflux from plastids and may contribute to the detoxification of excess Zn.

O*s*HMA2 is localized in the plasma membrane and transports Zn and Cd (Nocito et al., 2011; Satoh-Nagasawa et al., 2012; Takahashi et al., 2012b). The expression of *OsHMA2* was observed mainly in the roots, where *OsHMA2* transcripts were abundant in vascular bundles (Takahashi et al., 2012b). OsHMA2 could play a role in loading Zn and Cd into the xylem and participates in root-to-shoot translocation of these metals in rice. It also could participate in Zn transport during flowering and seed maturing.

4.3 ZIP transporters

'ZIP transporters' refers to ZRT-IRT-like Proteins, which were named for the two first members found, the high affinity yeast plasma membrane Zn uptake

transporter, ZRT1, and the high affinity *A. thaliana* plasma membrane Fe uptake transporter, IRT1 (Eide et al., 1996; Zhao and Eide, 1996). Subsequent studies of other plant ZIP family members demonstrated that not all the members of this family are involved in plasma membrane micronutrient uptake. The ZIP membrane proteins are involved in the transport of four essential micronutrients: Zn, Fe, Mn, and Cu (Eide et al., 1996; Grotz et al., 1998; Wintz et al., 2003; Cohen et al., 2004; Pedas et al., 2008; Lin et al., 2009). They act as influx carriers, participating in the uptake from the soil (similar to ZRTs in yeast). In plant roots, as in yeast, Zn enters the cell *via* ZIP proteins, which are divalent metal transporters (Grotz et al., 1998; Colangelo and Guerinot, 2006; Puig et al., 2007). ZIP family members have also been shown to transport heavy metals such as Cd, and may also play a significant role in how various heavy metals, both essential and toxic, are taken up and translocated throughout the plant (Guerinot, 2000; Pence et al., 2000; Rogers et al., 2000). ZIP proteins contain eight transmembrane (TM) domains and a histidine-rich variable loop between TM3 and TM4.

In *A. thaliana* 15 ZIP family members have been identified. In general, these transporters are highly expressed under conditions of Zn deficiency, whereas their expression decreases quickly when Zn is added to the media (Talke et al., 2006) although they could transport other metals. Six of the *Arabidopsis* ZIP genes complemented a yeast Zn uptake-deficient mutant, one was able partially to complement a yeast Fe uptake-deficient mutant, six ZIP family members complemented a Mn uptake-deficient mutant, and none complemented the Cu uptake-deficient mutant (Milner et al., 2013). The most characterized members of this family are the three *Arabidopsis* ZIP

transporters, AtZIP1(IRT1), AtZIP2(IRT2), and AtZIP3(IRT3), with AtZIP1(IRT1) being by far the most well studied based on its seminal role in root Fe uptake and transport (Eide et al., 1996; Rogers et al., 2000; Vert et al., 2001; Connolly et al., 2002; Vert et al., 2002; Lin et al., 2009; Vert et al., 2009; Milner et al., 2013). AtZIP1 is localized in the vacuole. Very little or nothing is known about the function of the other 12 *Arabidopsis* ZIPs.

AtZIP2 and *AtZIP4* complement growth defects of yeast Cu and Zn transport mutants (Grotz et al., 1998; Wintz et al., 2003) and their transcript expression is up-regulated in *Arabidopsis* by deficiency of Cu and Zn, but not of Fe. AtZIP1 may play a role in remobilizing Mn from the vacuole to the cytoplasm in root stellar cells, and may contribute to radial movement to the xylem parenchyma. AtZIP2, on the other hand, may mediate Mn (and possibly Zn) uptake into root stelar cells, and thus also may contribute to Mn/Zn movement in the stele to the xylem parenchyma, for subsequent xylem loading and transport to the shoot. Although the role of these proteins in plant Cu transport still requires further characterization, the preference that ZIP family members show for divalent metals suggest that ZIP2 and ZIP4 proteins may transport Cu^{2+} ions.

Expression of both *AtZIP1* and *AtZIP2* genes are localized in the root stele, although *AtZIP1* expression was also found in the leaf vasculature. It was also found that AtZIP1 is a vacuolar transporter, while AtZIP2 is localized in the plasma membrane. The ZIP4 family member is localized in the chloroplast (Guerinot, 2000). *AtZIP1* transcript levels increase in the roots as the *Arabidopsis* plant ages, and its expression decreases in the shoot during the same developmental time sequence. *AtZIP1* transcript levels were higher in the

roots under both Zn- and Fe- deficiency. Fe acquisition in *Arabidopsis* roots under Fe-deficiency mostly depends on AtIRT1, which is considered the major Fe transporter at the root surface in *A. thaliana*. On the other hand, root and shoot *AtZIP2* transcript abundance decreased in response to Zn, Fe, and Mn deficiency. However, the observed lower transcript levels under Zn and Mn deficiency suggest that AtZIP2 is not a primary transporter involved in Zn and Mn uptake from the soil under Zn- and Mn-limiting conditions. Six cDNA encoding ZIP family members have been identified in the model legume *Medicago truncatula* L., and have been tested for the ability to complement yeast metal-uptake mutants (López-Millán et al., 2004).

The exact mechanism of this regulation is still unknown. It has been shown that at least ZIP4 in *A. thaliana* is regulated by transcription factors of the basic-region leucine zipper (bZIP) family: bZIP19 and bZIP23. These factors bind to a ZDRE (zinc deficiency response element), which has been found not only in the upstream region of *ZIP4*, but also of *ZIP1*, *ZIP3*, *ZIP9* and *IRT3*. Therefore, it is reasonable to assume similar regulation for these ZIP transporters.

4.4 YSL transporters

The yellow stripe-like (YSL) transporters belong to the oligopeptide transporter (OPT) superfamily (Curie et al., 2001; Curie et al., 2009) and transport tri-, tetra-, penta- and hexapeptides (Yen et al., 2001). Although the 3D structure and topology of these transporters is still unknown structural modelling predicts between 11 and 16 transmembrane (TM) domains and loops between TM domains containing a higher number of charged residues, which may participate

in substrate recognition of metal-chelator complexes (Conte and Walker, 2012). The YSL proteins play an important role in the long distance transport of metals complexed with phytosiderophores (PS) and nicotianamine (NA) (Colangelo and Guerinot, 2006; Conte and Walker, 2012). They are responsible for primary uptake of Fe–PS complexes into plant roots. It has been reported that *ys1* mutants were defective in the uptake of Fe–PS and Zn–deoxymugineic acid (DMA) (von Wirén et al., 1994; 1996). The plants that use Strategy II for Fe uptake, which involves the synthesis and secretion of phytosiderophores (PS) to increase Fe solubility in the rhizosphere, typically take up Fe-PS complexes at the root epidermis by YSL transporters. Members of this family have been investigated in *Zea mays*, maize (ZmYS1) (Curie et al., 2001), *Hordeum vulgare,* barley (HvYS1) (Murata et al., 2006), *A. thaliana* (AtYS1, AtYS2, AtYS3), *O. sativa,* rice (OsYS1, OsYS14, OsYS15, OsYS16) (Inoue et al., 2009; Lee et al., 2009; Zheng et al., 2012) and *Brachypodium distachyon,* brachypodium (BdYS1A) (Yordem et al., 2011). ZmYS1 protein, which was the first member of this family characterized, accumulates in roots and leaves of Fe-deficient plants and transports Fe-PS. It is localized on the distal side of epidermal cells of the crown and lateral roots (Ueno et al., 2009). In the leaves, ZmYS1 is localized in mesophyll but not epidermal cells, implicating it in intracellular transport of Fe in maize (Ueno et al., 2009). ZmYS1 has been extensively characterized on a biochemical level indicating that it plays a role in the homeostasis of Cu, Zn, Ni or Mn (Conte and Walker, 2012). ZmYS1 has a broad substrate specificity and can transport Cu(II)–MA, Zn(II)–MA and Fe(II)–NA, but has a lower affinity for Ni(II)–MA, Mn(II)–MA and Co(II)–MA. The ortholog HvYS1 transporter is localized in the plasma membrane and it is more

strongly expressed in roots, where it is up-regulated by Fe deficiency but not by deficiency of Mn, Zn or Cu (Murata et al., 2006; Ueno et al., 2009).

A. thaliana has 8 predicted YSL proteins. AtYSL1, AtYSL2 and AtYSL3 have been studied is some detail. The *AtYSL1* gene was expressed in the xylem parenchyma of leaves, pollen and young siliques and it was induced by Fe excess in shoots. AtYSL2 is the most similar transporter to ZmYS1, and its transcript accumulates under conditions of Fe sufficiency or Fe resupply, and the transcript levels also respond to Cu and Zn (DiDonato et al., 2004; Le Jean et al., 2005; Schaaf et al., 2005). Localization of AtYSL2 in root endodermis and pericycle cells facing the xylem tubes has suggested its participation in lateral movement of Fe and/or Cu within the veins (Schaaf et al., 2005). These proteins seem to be involved in the unloading of metal-NA from vasculature into developing tissues, in immobilization of metal-NA from senescent leaves and in an efficient loading of metal-NA into seeds. AtYSL1 and AtYSL3 are up-regulated during leaf senescence and could function in delivery of Cu, among other metals, from vascular tissues, as well as in Fe-NA delivery to seeds (Waters et al., 2006). *AtYSL2* and *AtYSL3* are differentially expressed under metal deficiencies and their products can transport Cu^{2+}, Mn^{2+} and Fe^{2+} (Wintz et al., 2003). AtYSL4 and AtYSL6 are involved in managing chloroplastic Fe. They are localized in the vacuolar membrane and endoplasmic reticulum (Conte et al., 2013; Divol et al., 2013). *YSL4* and *YSL6* expression patterns support the physiological role of YSL4 and YSL6 in detoxifying Fe during plastid dedifferentiation occurring in embryogenesis and senescence.

The rice genome contains 18 putative *YSL* genes. *Os*YSL2 has been shown to transport Fe^{2+}-NA and Mn^{2+}-NA complexes but not Fe^{3+}-NA. A role in

the transport of divalent cations in the phloem has been suggested (Koike et al., 2004; Colangelo and Guerinot, 2006). The member most closely related to ZmYS1 is OsYSL15, which similarly is localized in the plasma membrane. It is up-regulated in roots under Fe deficiency and transports Fe(III)–DMA and probably Fe(II)–NA based on the *fet3fet4* complementation (Murata et al., 2006; Inoue et al., 2009). Over-expression of *OsYSL15* increases the Fe concentration in leaves and seeds. On the other hand, the tissue expression profile of *OsYSL18* suggests that its protein products are involved in translocation of Fe in reproductive organs and phloem in leaf joints (Aoyama et al., 2009). By contrast, OsYSL16 is a phloem-localized transporter involved in Cu-NA distribution and redistribution. It is present in the roots, leaves and un-elongated nodes at the vegetative growth stage. It has been suggested that OsYSL16 is required for delivering Cu to developing young tissues and seeds (Lee et al., 2009).

The genome of *Brachypodium distachyon* contains 19 protein homologs to ZmYS1, and two of them are most likely YS1 orthologs, BdYS1A and BdYS1B (Yordem et al. 2011). Studies on YSL genes have also been carried out in metal hyperaccumulator plants such as *Thlaspi caerulescens. TcYSL3* is expressed throughout the plant body, and *TcYSL5* and *TcYSL7* are expressed in shoots and the central cylinder in the roots. *TcYSL5* is highly expressed in shoots and *TcYSL7* is highly expressed in flowers (Gendre et al., 2007).

4.5 Copper chaperones

The Cu chaperones are low-molecular-weight metal-receptor proteins involved in the intracellular trafficking of metal ions. These proteins contain Cu-binding

domain(s), to assist Cu intracellular homeostasis by their Cu-chelating ability. The limited solubility and high reactivity of Cu^+ inside the cell requires the participation of these specialized proteins. Consequently, Cu chaperones bind and deliver Cu ions to intracellular compartments and insert the Cu into the active sites of specific partners, Cu-dependent enzymes (O'Halloran and Culotta, 2000; Huffman and O'Halloran, 2001). These proteins prevent inappropriate Cu interaction with other cellular components. *Arabidopsis thaliana* has at least three Cu chaperones, including the Cu chaperone for superoxide dismutase (SOD; CCS) and two homologs of yeast Antioxidant Protein1 (ATX1), the Copper Chaperone (CCH) and ATX1 (Casareno et al., 1998; Chu et al., 2005; Puig et al., 2007; Shin et al., 2012). CCH has been the most extensively studied of the Cu chaperones in plants (Mira et al., 2001a; 2001b). The CCH chaperone exhibits the conserved features of the ATX1-type metallochaperone family such as typical lysine residues, overall βαββαβ fold structure and a MxCxxC Cu^+-binding motif in the N-terminus (Pufahl et al., 1997). However, CCH also has a plant specific C-terminal domain with special structural characteristics (Mira et al., 2001a; Mira et al., 2001b; Mira et al., 2004). In *A. thaliana* the Antioxidant Protein1 (ATX1) and ATX1-Like Copper Chaperone (CCH) share high sequence homology. Both CCH and ATX1 chaperones complement the yeast *atx1* mutant and interact with the N-terminus of AtHMA5 (Andrés-Colás et al., 2006). However, the C-terminus of CCH has a negative effect on its interaction with AtHMA5. The plant *CCH* gene expression has been related to oxidative stress and senescence, when the plant reallocates nutrient resources. High levels of *CCH* expression were found in *Arabidopsis* stems and vascular cells that lack nuclei. A plant-specific role in Cu

symplasmic transport through the plasmodesmata during senescence, associated with nutrient mobilization, has been proposed for this extra C-terminus domain of CCH. Expression of *CCH* increases with oxidative stress, senescence, and Cu deficiency. The activities of antioxidant enzymes in *atx1* and *cchatx1* mutants were markedly regulated in response to excess Cu.

A CCH chaperone has been also identified by differential display in tomato (*Lycopersicon esculentum;* LeCCH) infected with the fungal pathogen *Botrytis cinerea* (Company and González-Bosch, 2003), suggesting an interesting relationship between Cu homeostasis and plant defense responses.

The COX17 chaperone shares sequence similarity to COX17 from yeast that may mediate the delivery of Cu to the mitochondria for the assembly of a functional cytochrome-c oxidase complex (Balandin and Castresana, 2002). In this manner COX17 would contribute to the increase in activity of specific enzymes that are required to preserve organelle functionality in a number of biotic and abiotic stress situations.

Despite their role in Cu homeostasis, neither CCH nor RAN1(AtHMA7) are induced by Cu treatment, indicating that they may be more important in helping cells cope with Cu deficit than Cu excess. In contrast, activation of *AtCOX17* gene expression in response to Cu treatment might be an indication of a function like metallothioneins, which are also induced by high concentrations of metals (Zhou and Goldsbrough, 1994). Nevertheless, further experimental support is necessary to establish the function of these proteins.

The *CCS* gene, homolog of the yeast *Ccs1p/Lys7p* gene, encodes a protein that delivers Cu to the Cu/Zn-SOD by a protein-protein interaction. It has been identified in tomato (*LeCCS*) (Zhu et al., 2000), *A. thaliana* (Wintz and

Vulpe, 2002; Chu et al., 2005), potato (*Solanum tuberosum; StCCS*) (Trindade et al., 2003), maize (*ZmCCS*) (Ruzsa and Scandalios, 2003) and soybean (*GmCCS*) (Sagasti et al., 2011). AtCCS has a predicted chloroplast targeting sequence, but dual localization in both cytosol and plastids (Chu et al., 2005). Therefore, it is possible that AtCCS delivers Cu to both cytosolic and chloroplastic Cu/Zn-SOD enzymes, perhaps using an alternative translation start site. It has been shown that AtCCS is Cu up-regulated and co-regulated with cytosolic and chloroplastic Cu/Zn-SOD targets, indicating an important role in the regulation of oxidative stress protection. An up-regulation of *AtCCS* mRNA has been also found in response to senescence. Additionally, AtCCS, and both cytosolic and chloroplastic Cu/Zn-SODs, were down-regulated in response to Cu deficiency. It has been also proposed that *AtCCS* expression is regulated to allow the most optimal use of Cu for photosynthesis (Shikanai et al., 2003).

StCCS gene expression is induced by auxin, which is known to play a role in different stages of plant development. Auxins have a promoting effect on cell elongation/expansion. Surprisingly, potato plants sprayed with $CuSO_4$ did not respond with a significant change in *StCCS* expression (Trindade et al., 2003). This is consistent with the inhibition of *StCCS* gene expression observed when potato plants were grown *in vitro* in media supplemented with 10 mM $CuSO_4$. This surprised finding may be explained if the presence of a chaperone would not be required for the incorporation of Cu in the Cu/Zn-SOD when Cu is present at high concentrations in leaves.

4.7 Regulation of copper proteins

The *Arabidopsis* genome encodes a 17-member zinc finger plant-specific transcription factor family named SPL (for SQUAMOSA-promoter binding-like proteins) (Birkenbihl et al., 2005). The most studied member of this family is SPL7, which has been shown to be essential for the transcriptional activation in response to Cu deficiency (Bernal et al., 2012). It mediates the substitution of Cu/ZnSOD by FeSOD1 in chloroplasts. SPL7 activates the expression of *FeSOD1* and promotes the degradation of Cu/ZnSOD mRNA through its binding to GTAC motifs within the promoter region of target genes (Yamasaki et al., 2009). Several Cu-regulated microRNAs such as *miR398, miR397, miR408 and miR857* have been described to be involved in the degradation of the mRNAs encoding cuproproteins (Yamasaki et al., 2007; Abdel-Ghany and Pilon, 2008). The regulation by *miRNA* is a widespread response to Cu deficiency. SPL7 also activates the Cu-responsive genes *COPT1*, *COPT2* and *COPT6* (Gayomba et al., 2013). The comparison of global responses to mild deficiency and excess Cu has been investigated in *A. thaliana* (Andrés-Colás et al., 2013) and regulatory elements in the promoter regions of the Cu-deficiency over-represented gene were proposed. The CuAtDB database lists the cuproproteins and Cu homeostasis factors identified (www.uv.es/cuatlab). Regulators that mediate the response to excess Cu are still known.

5 DISTRIBUTION AND SPECIATION OF COPPER IN PLANTS AND PLANT PARTS

The distribution and speciation of Cu in plants has been investigated using different techniques such as inductively coupled plasma mass spectrometry

(ICP-MS), electron dispersive X-ray spectroscopy (EDXS) (Monni et al., 2002; Ni et al., 2005), electron energy loss spectroscopy (EELS) (Turnau et al., 1993; Neumann et al., 1995; Lichtenberger and Neumann, 1997), X-ray absorption spectroscopy (XAS) (Tao et al., 2004), X-ray absorption fine structure (EXAFS), synchrotron-based micro X-ray fluorescence (μ-SXRF) (Punshon et al., 2013), micro X-ray absorption near edge structure (μ-XANES) (Shi et al., 2004; Song et al., 2013) and mass spectrometric imaging techniques (LA-ICP-MS imaging) (Wu and Becker, 2012; Wu et al., 2013). In the last few years, significant improvement has been seen in the usefulness of synchrotron based techniques and X-ray absorption spectroscopy for biological samples, including in the study of localization of metals and metalloids (West et al., 2012). Comparatively, it has been reported that synchrotron radiation X-ray fluorescence (SRXRF) microprobe is a more sensitive technique and is less injurious to cells (Song et al., 2013).

Cu normally is accumulated in root tissues and in particular in cell walls (Nishizono et al., 1987; Shi et al., 2008; Kopittke et al., 2011; Shi et al., 2011). For instance, in *Leymus chinensis*, Cu concentration varies between plant parts in the order of roots > rhizomes > stems > leaves > litter (Zhou et al., 2013). The dynamic distribution of nutrients during germination of *Brassica napus* seeds revealed a relatively rapid allocation of Cu to roots (Eggert and von Wirén, 2013). However, the results derived from these studies are mostly difficult to compare since Cu distribution can vary depending on the nature of chemical reagent used, the concentration applied, the time of exposure and the handling of samples (Kopittke et al., 2011). Long-term exposure may not give a real status of the initial metal uptake or toxicity. Differences in trace elements

distribution and speciation have been found between the use of fresh, frozen-hydrated, frozen-dried or oven-dried plant materials (Kopittke et al., 2011). Furthermore, the type of plant can also influence these kinds of analyses, differences can be found between a sensitive, tolerant or hyperaccumulator plant since in non-hyperaccumulator plants trace elements are in much lower concentrations. Studies on agronomic crops and vegetables are comparatively more limited than those referring to tolerant plants and hyperaccumulators (Callahan et al., 2006).

A comparison of Cu localization by EDXS in *Elsholtzia splendens* Nakai ex F.Maek., a Cu-tolerant plant growing in mine areas in the south of China, and the non-tolerant *Astragalus sinicus* L. showed that the majority of Cu in the tolerant plant was localized primarily in the cell wall and vacuole, but in the non-tolerant species Cu precipitates on the plasma-membrane, in the chloroplasts and cytoplasm under levels of Cu supply that were toxic to both species (Ni et al., 2005). The spatial distribution and speciation of Cu in different zones of the root tip and meristematic zone of cucumber (*Cucumis sativus* L.) have been investigated using μ-SRXRF and μ-XANES and freeze-dried samples (Song et al., 2013). Control roots exhibited a higher content of Cu in the root cap and the front of the meristematic zone. These are the most active regions for the absorption of trace elements (Walker et al., 2003). After exposure of roots to 100 μM Cu over 72 hours the distribution of elements, especially Cu and Fe, changed in these regions. The content of Cu in the root cap and meristematic zone increased sharply, but no accumulation was observed in the maturation and elongation zones. By contrast, Fe content decreased in these regions (Song et al., 2013). XANES analysis indicated that after 72 hours of treatment

Cu in the root tip bound mainly to alginate, citrate, and cysteine-like ligands, and little was deposited as CuO. Distribution dynamics indicated that Cu-alginate-like ligands were accumulated more as a proportion of the Cu bound with ligands with distance from the root cap to the maturation zone (from 25.7 to 71.2 %) whereas proportions of Cu bound with citrate-like ligands decreased along the same direction (from 53.1 to 17.7 %). The cysteine-Cu-like species gradually increased in proportion from the root cap to the elongation zone (from 17.7 to 28.7 %) but this proportion sharply declined in the maturation zone (5.1 %). CuO-like species only accounted for a small proportion, but also tended to increase from the root cap to the maturation zone (from 3.4 to 6.0 %). In *Commelina communis* L., a Cu-tolerant plant that grows in Cu mine areas, the metal distribution after 100 μM Cu treatment was analyzed by SRXRF (Shi et al., 2011). A high level of Cu was found in the root meristem and epidermis, it being lower in the cortex than in the vascular cylinder. In the cross section of elongation tissue Cu concentration decreased from the epidermis to the endodermis and reached the highest level in the vascular cylinder. *In situ* analysis with μ-SRXRF of hydrated roots of cowpea (*Vigna unguiculata* (L.) Walp.) after 24 hours of exposure to copper revealed that Cu was located mostly in the rhizodermis (cell wall) and the outer cortex bound to polygalacturonic acid (60%), which is the skeleton component of pectin, and its concentration was substantially lower within the inner cortex and stele. However, after only a short time (3 hours) of exposure Cu was mainly associated with cysteine (57%) or citric acid (43%) (Kopittke et al., 2011). In comparison, the distribution of Zn was different, showing the highest content in the meristematic region and the lowest content in the cortex. In roots, stems,

and leaves of *E. splendens*, a Cu-tolerant plant, most Cu was bound to O-containing ligands in the cell wall when plants were grown in 300 μM Cu for 10 to 60 days (Shi et al., 2008). Similarly, in *Crassula helmsii* (Kirk) Cockayne, a Cu-tolerant amphibious water plant, Cu was accumulated in its shoots bound almost exclusively by oxygen ligands, like organic acids, with no contribution of sulfur ligands or Cu-Cu interactions (Küpper et al., 2009). This finding contrasts with observations in non-accumulator plants (Mijovilovich et al., 2009). It is thought that Cu is bound by weak ligands (*i.e.,* sulfur ligands) in Cu-hyperaccumulator plants like *Crassula helmsii* but is bound by strong ligands (*i.e.,* O-ligands) in Cu non-accumulators such as *Thlaspi caerulescens*.

The distribution of Cu in leaf epidermis, and cross-section of the stem of *E. splendens*, was analyzed by μ-SRXRF (Shi et al., 2004). The Cu concentration in leaves of this species reached 1000 mg kg^{-1} in solution culture (Yang et al., 2002). The highest Cu levels were measured in the vascular tissues of the stems and petioles, while Cu levels in the mesophyll were higher than in the leaf epidermis. A significant correlation between distribution of Cu and P, S, and Ca was observed, which suggested P, S, and Ca can play an important role in Cu accumulation in *E. splendens*. Based on the significant correlation between Cu and distribution of the elements Mn, Fe, and Zn, it seemed that Cu, Mn, Fe, and Zn could be transported by the same transporters, with these having a broad substrate range.

A comparison between Cu ions and Cu-chelate was assayed in *Brassica carinata* A.Braun plants. Chelates make Cu more available for plant uptake and translocation to the shoots. The plants were treated with 30 μM or 150 μM $CuSO_4$ or Cu-EDDS ((S,S)-*N*,*N*′-ethylenediamine disuccinic acid) in hydroponic

solution and Cu distribution was determined by micro-proton induced X-ray emission (Cestone et al., 2012). Differences depending on Cu concentrations were found between both treatments. In roots, the 30 µM treatments with both $CuSO_4$ and Cu-EDDS resulted in higher Cu concentrations in epidermal/cortical regions. With the 150 µM $CuSO_4$ treatment, the Cu was mainly concentrated in the vascular tissues, indicating increased uptake into the symplast and further into the xylem. Similar effects were not observed in the 30 µM Cu treatment, supporting the concept that this treatment was not as harmful to the root membranes (Cestone et al., 2010), and thus a lower level of passive symplast Cu influx was maintained. With 150 µM Cu-EDDS, the highest Cu concentration was detected in the endodermis and the adjacent inner cortical cell layer, which indicates that the endodermis prevented the translocation of Cu into the vascular tissues, and that an efficient apoplastic barrier was still preserved, in spite of the high root Cu concentrations. The incubation of plants with 150 µM Cu-EDDS enhanced metal translocation to shoots, in comparison with the corresponding $CuSO_4$ treatment. The transport of Cu-EDDS was active and dependent on ATPase since inhibition of H^+-ATPase activity resulted in a reduction of Cu accumulation in 30 µM Cu-EDDS-treated roots and 150 µM Cu-EDDS-treated leaves, and induced changes in Cu distribution in the leaves. This indicates that active mechanisms are involved in retaining Cu in the leaf vascular tissues.

6 INTERACTION OF COPPER WITH UPTAKE OF OTHER ELEMENTS

Metal ions can bind to organic ligands in a metal-binding site of a metalloprotein, metal-chaperone or metal transporter with different affinities

(Fraústo da Silva and Williams, 2001). These differential metal binding affinities are determined by diverse factors such as their different chemical properties (*i.e.,* redox potential, coordination geometry, charge and thermodynamic and kinetic properties of ligand exchange), the size of metal binding-site cavity in a protein and the geometry of ligand atoms, among others. Accordingly, in a given metalloenzyme, a specific metal ion is used for a specific function. However, according to the Irving-Williams series ($Zn^{2+} < Cu^{+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$) cations chemically similar to each other can compete in uptake pathways. Thus, one major mechanism of toxic action of all transition metal in plants is the efficient competition of metal ions for specific binding sites (Fraústo da Silva and Williams, 2001; Yruela, 2008). For instance, the central ion Mg^{2+} in chlorophyll can be substituted by Cu and other toxic metals under metal excess conditions, resulting in an impairment of photosynthesis (Küpper and Kroneck, 2005).

It has been discussed that metal toxicity may be caused by a disturbance of nutritional balance, resulting in a deficiency of essential elements, which increases toxicity. Antagonistic interactions between essential micro- and macro- nutrients and non-essential elements can take place. Cu and Fe antagonism often occurs in plants grown under Cu toxicity (Foy et al., 1978; Wallace and Cha, 1989; Lombardi and Sebastiani, 2005). Excess Cu in hydroponic medium induces an Fe-deficiency in bean plants (Pätsikkä et al., 2002). In *Commelina communis* Fe-deficiency induces Cu accumulation (Chen et al., 2004). In leaf blades of sugar beet grown hydroponically Fe-deficiency increases the Cu content and decreases the Zn content (Rombolà et al., 2005). In *Phaseolus vulgaris* L. plants concentrations of Fe, Zn, and K were reduced

significantly simultaneously when $CuSO_4$ was administrated (Bouazizi et al., 2010). Cu competed with Fe, Mn and Zn uptake in the Mn-hyperaccumulator *Phytolacca americana* L. plants supplemented with 25 µM Cu (Zhao et al., 2012). Zn decreased concentrations of micro- and macroelements such as Fe, Mg, Mn, Ca and K. Another interesting aspect is that micronutrient interactions can vary depending on how they are supplied. Cu can interact differently with Fe and Zn depending on whether excess Cu is supplied through a foliar treatment or in a hydroponic medium (Bernal et al., 2007a). Soybean plants showed no antagonist interaction between Cu and Fe when excess Cu was supplied through leaves, but Cu competed with Fe-uptake in plants grown with excess Cu in the hydroponic medium. Concerning Zn-uptake, soybean plants exhibited a decrease in Zn content upon Cu treatment of leaves whereas the opposite was observed upon Cu supply through the roots. The different plant response observed following these two Cu-treatments might be explained assuming different Cu-uptake strategies in leaf and root cells or different compartmentalization mechanisms that prevent the metals from being transported.

In metal-polluted areas toxic metal ions can enter into most plants, since the metal homeostasis network is not equipped to avoid the entry of non-essential metal transitions at high concentration. The Mn and Zn content of roots decreased in *Pinus pinea* L.*, Pinus pinaster* Ait. and *Fraxinus angustifolia* Vahl at increasing Cu and Cd concentrations (Arduini et al., 1998). The absorption and translocation of different elements can be conditioned by the total metal composition in the soil. For instance, the co-occurrence of metals such as Cu, Zn, and Cr resulted in a greater reduction of the biomass of the

hyperaccumulator *Sesbania virgata* (Cav.) Pers. than the presence of a single metal, suggesting a synergistic or additive response. In the binary mixture of Cu and Zn, *Sesbania* plants absorbed the highest concentrations of these metals. In contrast, Cr was more absorbed in the individual treatments (Branzini et al., 2012). In particular, the highest concentration of Cu in both shoot/leaves and roots was observed when Zn was added simultaneously at high doses. The simultaneous presence of Cu and Zn increases the extraction capacity of *S. virgata plants*, indicating synergistic effects between them. This finding is in agreement with observations by Luo and Rimmer (1995), who demonstrated that the increase in Zn uptake due to the addition of Cu is approximately 20% and that Cu uptake also increases with addition of Zn. Total Zn concentration had a pattern of variation different from that of Cu. At high doses, the Zn concentration in shoots/leaves was higher than at low doses only with Cu. In contrast, when Cr was in binary mixtures, the concentration in roots was lower (Branzini et al., 2012). Cr was more absorbed in the individual treatments, suggesting a possible antagonistic relationship between the mixture constituents. A possible explanation for this trend could be that the sorption capacity of each metallic cation of the mixture might decrease in competitive processes (Flogeac et al., 2007). Accumulation of metals in *Sesbania drummondii* (Rydb.) Cory seedlings was dependent on the combination of metals in the medium (Israr et al., 2011). *S. drummondii* plants can accumulate higher concentrations of metals such as Pb, Cu, Ni, and Zn, but the uptake of these elements is affected not only by the elements in single applications, but also by the combinations of the elements. For all different combinations of metal accumulation studied with *S. drummondii* seedlings, bioaccumulation of a single

metal in the roots as well in the shoots was affected by the presence of a second metal, resulting in the inhibition or increase in the bioaccumulation of one metal over another. Uptake of a single metal by *S. drummondii* was affected by the presence of a second metal, suggesting an antagonistic effect or competition between metals at the plant uptake site (Israr et al., 2011). The uptake of metals followed the order Pb>Cu>Zn>Ni in roots and Pb>Zn>Cu>Ni in shoots.

7. DIAGNOSIS OF COPPER STATUS IN PLANTS

A preliminary diagnosis of nutrient status can be carried out by the observation of changes in the appearance of the leaves. The lack of green color in leaves is a symptom associated with alterations in Cu status either by deficiency or toxicity, but can be caused also by changes in other nutrients. Morphological changes in leaves and roots are also indicative of nutrient alterations. The visual method is not diagnostic for any specific nutrient stress symptoms but is an extremely valuable tool for the rapid evaluation of the nutrient status of a plant. The main advantage of the visual diagnostic symptoms is that they are readily observed and provide an immediate evaluation of nutrient status. However, the fact that these visual symptoms do not develop until after there is a major effect on growth and development constitutes a disadvantage. More precise tools include microscopic studies, spectral analysis, tissue and soil analysis and enzymatic assays. These methods all vary in their precision, rapidity and their ability to predict future Cu status. Enzymatic analysis normally gives a more specific diagnosis *(i.e.*, the determination of the CuZnSOD activity).

It is known that Cu and Fe uptake are balanced and in equilibrium within the chloroplast in order to preserve the photosynthetic process. The chloroplast enzymes involved in the defense against reactive oxygen species (ROS), Fe-SOD and Cu/ZnSOD, are synthesized depending on the Cu status. Cu/Zn-SOD and Fe-SOD are down-regulated and up-regulated in response to Cu deficiency, respectively (Abdel-Ghany and Pilon, 2008; Pilon et al., 2011; Andrés-Colás et al., 2013). The down-regulation of the chloroplast Cu/Zn-SOD upon occurrence of low Cu content contributes to maintaining a Cu pool for plastocyanin, allowing plants to save Cu for essential functions such as photosynthetic electron transport (Yamasaki et al., 2007). The availability of Cu affects Cu/Zn-SOD enzymes, diminishing their expression and activity. Therefore, the analysis of these proteins and enzymatic activity levels or their transcript expression provides information of the Cu status of the plant. These chloroplastic enzymes can be considered as markers of Cu demand.

The chloroplast Ca^{2+} transducer *CAS* gene and the gene for the flavoprotein subunit of succinate dehydrogenase in mitochondria *SDH1-2* have been also proposed as markers for Cu deficiency and excess Cu, respectively (Andrés-Colás et al., 2013).

8. FORMS AND CONCENTRATIONS OF COPPER IN SOILS, AVAILABILITY TO PLANTS

The average concentration of total Cu in soils ranges from 2 to 200 mg kg^{-1} (Mortvedt, 2000). Distribution of Cu concentrations in soils around the world have been reviewed (Kubota, 1983; Adriano, 1986; Kabata-Pendias and Pendias, 1992; Kopsell and Kopsell, 2006). Kastanozems, Chernozems,

Ferrasols, and Fluvisols contain the highest levels of copper, whereas Podzols and Histosols contain the lowest levels. Plants cannot usually take up the total amount of a metal micronutrient present in the growth medium or soil. Only the soluble fraction of soil solution, where Cu can exist as ionic species or complexed to soluble compounds, is directly available for plant uptake, while metal-precipitates, complexes with organic matter, metals adsorbed on clays, oxides, and the matrix of soil minerals are less available (Barber, 1995). The proportion of total metal which is in the soil solution is determined by factors such as pH, organic matter, clay and redox conditions. The fraction of the metal which plants can absorb is known as the available or bioavailable fraction.

Cu is present as sulfide minerals, stable oxides, silicates, sulfates and carbonates (Krauskopf, 1972; Mortvedt, 2000). The most abundant copper-containing mineral is chalcopyrite ($CuFeS_2$). The redox conditions of a soil can play a role in the availability of Cu. The redox status of the soil can be affected by many factors including waterlogging and compaction (Patrick and Jugsujinda, 1992; Evangelou, 1998). High P levels can alter the surface properties of soil colloids, possibly resulting in a redistribution of trace metals among various forms in soils.

8.1 Distribution of copper in soils

The pH in culture solution and soils affects Cu speciation, solubility, complexation and adsorption (Payne and Pickering, 1975; Msaky and Calvet, 1990; Reddy et al., 1995). However, some soil studies have found little relationship between soil pH and Cu concentration in the soil solution (Jeffery and Uren, 1983; McGrath et al., 1988; Sauvé et al., 1997). The reason for this is

the strong affinity of Cu for organic matter (Norvell, 1991). Therefore, the amount of organic matter dissolved in the soil solution, especially in soils with high organic matter content, can be a more important determining factor on Cu solubility than pH. Cu ions form strong coordination complexes with organic matter, altering their availability to plants (Stevenson, 1976; Stevenson, 1991). Hence, Cu is often predominantly found bound to the organic matter fraction in the soil and soil organic matter can be the most important soil factor in determining Cu bioavailability (del Castilho et al., 1993). In a Chernozem, between 37 and 91% of the total soil Cu was present in the organic fraction depending on level of Cu contamination (Pampura et al., 1993). In a range of Cu-contaminated soils greater than 98% of the Cu in the soil solution was bound to organic complexes, irrespective of pH (Sauvé et al., 1997). Also, in a different range of soils, approximately 95% of soil solution Cu was complexed, irrespective of pH (Fotovat et al., 1997). Reddy et al. (1995) found the proportion of Cu bound to organic matter in the soil solution increased from 37 to 95% as the pH decreased. In addition, Cu applied as sewage sludge was retained in the soil solution in greater quantities than Cu applied as a sulfate because it was bound to dissolved organics from the sludge (Miller et al., 1987) and the activity of the highly available Cu^{2+} ion has been inversely correlated with soil organic matter (McBride et al., 2003).

9 SOIL TESTING

Soil testing methods can be used to predict the soil capacity to provide essential micronutrients to a crop. These methods were mostly developed more than 40 years ago and have changed little in recent decades, despite changing

demands. They are based on a chemical extraction of the soil, which reflects the levels of nutrients which will be available to the plant during its growth, combined with a determination by using atomic absorption spectrometry (AAS) or inductively coupled plasma mass spectrometry (ICP-MS). Historically, soil testing was developed to determine if soil nutrients were deficient or adequate. However, more recently they are being extended to evaluate excess or toxic levels of nutrients and trace elements. Unfortunately, in these cases the validation of soil tests presents limitations.

Various extraction methods have been used to assess the availability of metals in soils. In particular, for Cu the most commonly chemical reagents used are acids like 0.1 mol L^{-1} HCl, complexing solutions such as Mehlich-1, Mehlich-2 and Mehlich-3 (Mehlich, 1984), diethylenetriaminepentaacetic acid (DTPA), (Lindsay and Norvell, 1978), and salts like sodium acetate trihydrate (Morgan, 1941) ammonium acetate (McIntosh, 1969; Ure et al., 1993), or 0.01 mol L^{-1} $CaCl_2$. Mehlich-3 is composed of 0.2N CH_3COOH, 0.25N NH_4N0_3, 0.015N NH_4F, 0.013N $HN0_3$, 0.001M EDTA. It has been demonstrated that in comparison with Mehlich-2, the combination of EDTA and acids increases Cu extraction by 170% (Mehlich, 1984). The DTPA extractant consists of 0.005M DTPA, 0.1M triethanolamine, and 0.01M $CaCl_2$, with a pH of 7.3.

de Abreu et al. (1996) compared the efficiency of several reagents in the determination of available Cu in 31 soils from the State of Sao Paulo, Brazil using wheat and beans as test plants. The results showed that the extraction of the available Cu decreased in the order: Mehlich-3 > TEA-DTPA > Mehlich-1. In pasture soils from New Zealand the efficiency of various chemical reagents in extracting the Cu from the soil followed the order: TEA-DTPA > Mehlich-3 >

Mehlich-1 > 0.02 M $SrCl_2$ > 0.1 M HCl > 1.0 M NH_4NO_3 > 0.01 M $CaCl_2$ > 0.1 M $NaNO_3$ > 0.01 M $Ca(NO_3)_2$ (Khan et al., 2005). Field experiments carried out in a Typic Eutrorthox have also indicated that Mehlich-3 yields the highest concentrations of Cu (Nogueirol et al., 2013). Testing experiments in acidic soils of Venezuela using five Cu reagents, DTPA, DTPA-HCl, EDTA, HCl, and Mehlich-1, in a greenhouse experiment with corn as a test crop indicated that DTPA-HCl and DTPA extractants could be a useful for producing an index for predicting Cu deficiency in acidic soils (Rodríguez and Ramírez, 2005).

The main difficulties in choosing a soil test derive from the extraction capacity of the reagent, since a substantial fraction of the extracted element may not be available for plants, especially when aggressive reagents are used. On the other hand, the proportion of Cu that can be extracted by soil test extractants can vary with the soil matrix (Khan et al., 2005). McBride *et al.* (McBride et al., 2009) have reported that the fraction of total Cu and Zn extracted by aggressive tests (*i.e.* Mehlich-3, DTPA) was much higher than the fraction extracted by $CaCl_2$, with the Morgan tests being intermediate. Less aggressive reagents, such as 0.01M $CaCl_2$, are considered frequently better for predicting plant availability of excess trace elements in soils than the traditional aggressive tests (Lebourg et al., 1996; Houba et al., 2000; McBride et al., 2003; Meers et al., 2007; Menzies et al., 2007). However, success in prediction of plant uptake by dilute $CaCl_2$ tends to diminish as the diversity of soils included in the analysis increases (McLaughlin et al., 2000). This is a problem encountered with all soil tests and presumably reflects the various biological, chemical, and physical factors that influence plant uptake that are not measured in the laboratory soil test itself. The main disadvantage of a non-aggressive

reagent such as 0.01 M $CaCl_2$ is that it yields low extractable concentrations for some trace metals, particularly when soil metal concentrations are at or near background levels.

The accumulation of organic matter in soils can affect the efficiency of chemical extractants by causing structural and chemical alterations including ion exchange reactions, reactions with soil minerals, increased liberation of organic anions, complexing of metallic cations, and oxidation–reduction reactions. Certain investigations have demonstrated that extractants developed for predicting positive crop responses to added fertilizer do a poor job of predicting toxicity or excessive uptake (Nogueirol et al., 2013). Accordingly, the soil tests have difficulties when sewage sludge has been applied for several years to crops, since extractants were originally developed for conventional systems and not to predict environmental risks. It has been observed that the efficiency of Cu and Zn extraction from field-contaminated soils was much lower than that from laboratory-spiked aged soils. For Mehlich-3 and DTPA tests, Cu and Zn in field-contaminated soils were less extractable by a factor of about 2 compared with the spiked soils. For less aggressive tests, the difference in extractability was even greater. It has been suggested that chemically non-aggressive neutral salts may be the most appropriate extractants where phytotoxicity is the concern in metal-contaminated soils (McBride et al., 2009).

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TABLE AND FIGURE LEGENDS

Table 1.- Copper transporter proteins in plants.

Table 2.- Copper concentrations in crop plants

Figure 1.- Scheme of transport pathways identified for Cu in a generic plant. Arrows indicate the proposed direction for metal transport. CCH, copper chaperone; ATX1, antioxidant 1; CCS, copper chaperone for Cu/Zn superoxide dismutase; COPT, copper transporter; ERT1, endoplasmic reticulum; HMA, heavy metal P-type ATPase; MA, mugineic acid; NA, nicotianamine; PAA, P-type ATPase of *Arabidopsis*; RAN1, responsive-to-antagonist 1; SOD, superoxide dismutase; YSL, yellow stripe-like protein; ZIP, IRT-like protein.

TABLE 1. Copper transporter proteins in plants

Family	Name	Description	Subcellular localization	Tissue expression	Code	References
COPT	AtCOPT1 OsCOPT1	High-affinity Cu^+ transporter	Plasma membrane	root apex, lateral root, embryo, trichomes, guard cells, pollen grains	At5g59030 Os01g0770700	1, 2, 3, 4, 5 6
	AtCOPT2 OsCOPT2	High-affinity Cu^+ transporter	Plasma membrane	leaves (high), roots, stems and flowers	At3g46900 Os05g0424700	2, 4, 7 6
	AtCOPT3 OsCOPT3	High-affinity Cu^+ transporter	Plasma membrane	stems (high), leaves and flowers (low)	At5g59040 Os01g0770800	2 6
	AtCOPT4 OsCOPT4	High-affinity Cu^+ transporter	Plasma membrane	oots (high), leaves (low), stems and flowers	At2g37925 Os03g0370800	2 6
	AtCOPT5 OsCOPT5	High-affinity Cu^+ transporter	Secretory pathway	leaves (high), stems, roots, flowers (low)	At5g20650 Os09g0440700	2, 8, 9 6
	AtCOPT6 OsCOPT6	High-affinity Cu^+ transporter	Plasma membrane	leaves (high)	At2g26975 Os04g0415600	10 6
	OsCOPT7	High-affinity Cu^+ transporter			Os09g0440700	6
ZIP	AtZIP1 OsZIP1	Divalent cation transporter	Plasma membrane	roots roots, leaves	At3g12750 Os01g0972200	11, 12, 13, 14
	AtZIP2 OsZIP2	Divalent cation transporter	Plasma membrane	roots	At5g59520 Os03g0411800	11, 12, 15
	AtZIP3 OsZIP3	Divalent cation transporter	Plasma membrane	roots	At2g32270 Os04g0613000	11
	AtZIP4 OsZIP4 MtZIP4	Divalent cation transporter	Thylakoid membrane	Leaf, chloroplasts Roots, shoots (phloem) Root, leaf	At1g10970 Os08g0207500 Q6VM18	11, 12 16 17
P_{1B}-ATPase	AtHMA1	Cu^{2+}-P_{1B}-ATPase transporter	Chloroplast envelope	Root, shoot	At4g37270	18, 19
	AtHMA5	Cu^+-P_{1B}-ATPase transporter	Plasma membrane	Root, flower, pollen	At1g63440	20
	AtHMA6(PAA1)	Cu^+-P_{1B}-ATPase transporter	Chloroplast envelope	Root, shoot	At4g33520 At5g44790	21, 22 23, 24

	AtHMA7(RAN1) OsHMA9 BnHMA7(BnRAN1)	Cu^{+}–P_{1B}–ATPase transporter	Trans–Golgi network	Root, shoot, leaf Vascular tissue (phloem xylem,), anthers		25, 26 27
	AtHMA8(PAA2) GmHMA8	Cu^{+}–P_{1B}–ATPase transporter	Thylakoid membrane	Shoot, leaf Leaf	At5g21930	22, 28 29
Chaperones	AtCCH LeCCH	ATX1–like Cu chaperone	Cytosol	Stem, vascular tissue		30, 31 32
	AtATX1	ATX1–like Cu chaperone	Cytosol	Root	At1G66240	20
	AtCCS LeCCS StCCS ZmCCS GmCCS	Chaperone for Cu/ZnSOD	Cytosol, chloroplast	Leaf	At1g12520 Q9ZSC1 Q6XZF8 Q9BBU5	21, 33, 34 35 36 37 38
	AtCOX17	COX17–like Cu chaperone	Mitochondria		At3g15352	33, 39
YSL	AtYSL1 ZmYS1 HvYSL1 BdYSL1A, BdYSL1B OsYSL1	Fe^{3+} –phytosiderophore/ NA–transporter	Plasma membrane	Leaf, shoots, pollen	At4G24120 Q9AY27 J7QZU1 Os01g0238700	40 41, 42, 43 42, 44 45 46, 47, 48
	AtYSL2 OsYSL2	Fe^{3+}/Cu^{2+}–NA transporter	Plasma membrane	Leaf, roots, shoots (low), pollen, petals, sepals	At5g24380 Os02g0649900	12, 49, 50 51, 52
	AtYSL3 TcYSL3	Fe^{3+}/Cu^{2+}–NA transporter	Plasma membrane	Leaf, anther, pollen	At5g53550 Q2XPY3	12, 53 54
	AtYSL4 OsYSL4	metal–NA complex transporter	Vacuolar membrane, endoplasmic reticulum	Leaf	At5g41000 Os05g0252000	55, 56
	AtYSL6 OsYSL6	metal–NA complex transporter	Vacuolar membrane, endoplasmic reticulum	Leaf	At3g27020 Os04g0390500	56
	AtYSL7 OsSYSL7	metal–NA complex transporter	Plasma membrane		At1g65730 Os02g0116300	57
	AtYSL8 OsYSL8	metal–NA complex transporter	Plasma membrane		At1g48370 Os02g0116400	57
	OsYSL14	metal–NA complex transporter	Plasma membrane	Leaf, roots (low)	Os02g0633300	46. 47. 48
	OsYSL15	metal–NA complex	Plasma membrane	Roots, shoots (low)	Os02g0650300	46

	OsYSL16	transporter metal–NA complex transporter	Plasma membrane	Roots	Os04g0542800	47
	OsYSL18	metal–NA complex transporter	Plasma membrane	Leaf, reproductive organs	Os01g0829900	58

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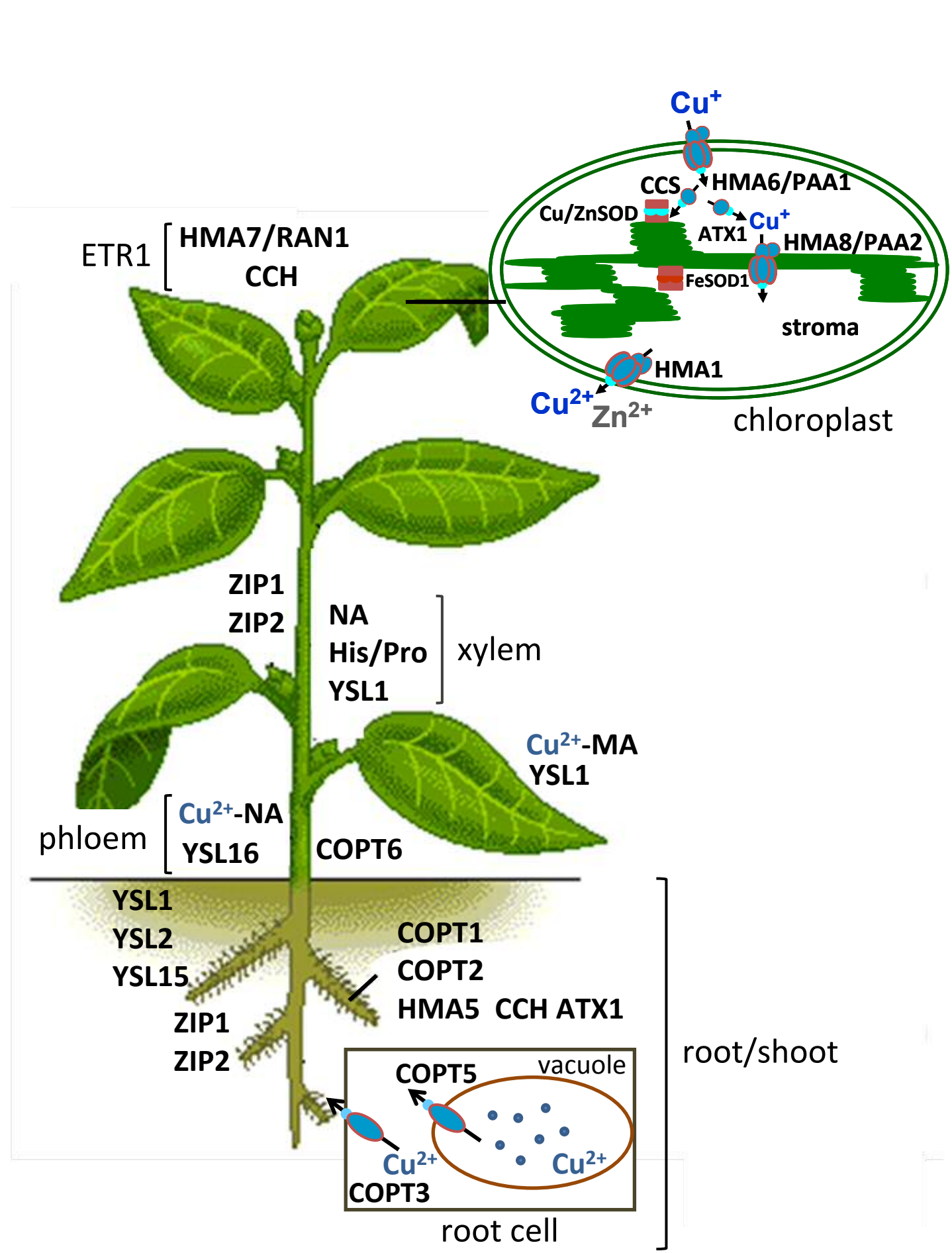
Table 2. Copper concentrations in crop plants

Crop species	Plant part	Cu Concentration (mg kg^{-1} dry matter)	Reference
Alfalfa (*Medicago sativa* L.)	Aerial parts	8.8	Kubota (1983)
Bluegrass (*Poa* sp.)	Aerial parts	5.5	Kubota (1983)
Clover, Ladino (*Trifolium repens* L.)	Aerial parts	7.9	Kubota (1983)
Clover, Red (*Trifolium pratense* L.)	Aerial parts	10.0)	Kubota (1983)
Durum wheat (*Triticum turgidum durum* L.)	Roots Roots of plants showing interveinal chlorosis due to excess Cu supply Shoots Shoots of plants showing interveinal chlorosis due to excess Cu supply	11-705 128-705 6-39 11-39	Michaud et al. (2007)
Fescue (*Festuca* sp.)	Aerial parts	4.4	Kubota (1983)
Lettuce (*Lactuca sativa* L.)	Whole plant	23-235	Ginocchio et al. (2002)
Maize (*Zea mays* L.)	Roots Roots of plants with Cu supply that gives lower biomass	32 299-7790	Ouzounidou et al. (1995)
	Roots Aerial parts	23-584 7-17	Brun et al. (2001)
	Leaves Stems Grains	10-21 8-12 2.5-3	Guo et al. (2010)
Oilseed rape (*Brassica napus* L.)	Roots Shoots	52-107 9-14	Chaignon et al. (2002a)
Onion (*Allium cepa* L.)	Whole plant	8-45	Ginocchio et al. (2002)
Orchard grass (*Dactylis glomerata* L.)	Aerial parts	5.2	Kubota (1983)
Perennial ryegrass (*Lolium perenne* L.)	Roots Shoots	14-42 3.2-9.5	Jarvis and Whitehead (1981)
Timothy-grass (*Phleum pratense* L.)	Aerial parts	4.6	Kubota (1983)
Tomato (*Solanum lycopersicum* L.)	Roots Shoots	48-157 6-10	Chaignon et al. (2002a)
	Whole plants	15-92	Ginocchio et al. (2002)
	Roots Shoots	14-42 3-7	Cornu et al. (2007)


Cu+
CCS
HMA6/PAA1
Cu/ZnSOD
Cu+
ATX1
HMA8/PAA2
FeSOD1
stroma
HMA1
Cu2+ Zn2+
chloroplast
ETR1
HMA7/RAN1
CCH
ZIP1
ZIP2
NA
His/Pro
YSL1
xylem
Cu2+-MA
YSL1
phloem
Cu2+-NA
YSL16
COPT6
YSL1
YSL2
YSL15
COPT1
COPT2
HMA5 CCH ATX1
ZIP1
ZIP2
COPT5
vacuole
Cu2+
Cu2+
COPT3
root cell
root/shoot